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The peroxisomal enzyme catalase protects aerobic organisms from free radical damage by converting hydrogen peroxide (H_2O_2) to molecular oxygen and water before it can decompose to form the highly reactive hydroxyl radical. In this manner catalase plays a central role in protecting against cellular oxidative damage. In humans, changes in catalase activity have been implicated in aging and in a number of disease states including cancer. We hypothesized that reduced catalase could potentially lead to an excess of H_2O_2 produced by peroxisomal oxidative reactions, which may then leak into the cell and cause DNA damage. To test this hypothesis we are examining the levels of catalase and peroxisomal fatty acyl-CoA oxidase in a variety of human breast samples and in breast cancer cell lines and in normal tissue and non-immortalized cells in culture. Catalase and acyl-CoA oxidase activities were extremely low in the human breast tumor samples therefore we have established conditions for using the polymerase chain reaction to quantitate the expression of the genes encoding peroxisomal catalase and acyl-CoA oxidase in a variety of breast cancer cell lines and in normal breast epithelial cells.

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Introduction

Peroxisomes are organelles that play an essential role in cell respiration and lipid metabolism. They contain several hydrogen peroxide-producing oxidases, and catalase which decomposes the hydrogen peroxide to water and molecular oxygen. The presence of peroxisomes has been confirmed in all human tissues looked at using cytochemical and/or biochemical techniques. There is very little information on peroxisomes and their functionality in human tumorous tissues, however reduced levels of catalase activity have been reported in colon carcinomas [1] human liver tumors [2] and in human epitheliomas [3]. Concerning human breast tissue, a study was performed in 1992 demonstrating that the specific activities of some peroxisomal enzymes were diminished in tumoral breast epithelial cells when compared to controls [4]. These investigators demonstrated that catalase activity in breast neoplastic tissues belonging to grade III (most fully progressed) were significantly lower than in adjacent normal tissues, while no differences were found in the number of peroxisomes. In this study the activities of some peroxisomal oxidases (including fatty acyl-CoA oxidase) were reduced, while others were unchanged. A simultaneous deficiency in peroxisomal ß-oxidation and of catalase activity could lead to the over-production of lipid peroxides, which are also implicated in carcinogenesis [5,6].

At the onset of our study the information available regarding the fluctuation in peroxisomal enzyme activities in humans raised the possibility that low catalase levels could be a factor in DNA damage and thus, in tumor formation or progression. There was no information regarding the relationship of the three proteins we proposed to study (catalase, acyl-CoA oxidase and the peroxisome proliferator-activated receptor (PPAR)) in human tumors. Breast tumors are an ideal model for us to test the hypothesis that variations in the ratios of these proteins may be involved in the tumorigenic process.

Our goal is to understand the role of peroxisomal metabolism in human mammary tissue. Initially we decided to concentrate on measuring the activities of catalase and acyl-CoA oxidase in human normal and tumorigenic breast tissue in order to understand whether there is any systematic correlation between the activities of these enzymes and the degree of tumorigenicity. We intend to also measure the expression levels of hPPAR in these tissues in order to determine whether hPPAR levels correlate with acyl-CoA oxidase activity. We would expect this to be the case if this receptor is regulating acyl-CoA oxidase in human mammary tissue. We will further determine whether there is any correlation between hPPAR expression and tumor formation. By investigating these parameters we intend to establish their possible involvement in breast tumor initiation/progression.

Experiments and Results

1) To determine levels of catalase in human breast tissue and human breast tumor samples.

To test the hypothesis that low catalase levels in combination with normal or increased levels of peroxisomal oxidative enzymes may lead, or contribute, to DNA damage in breast tumors our initial approach was to measure catalase activity in normal breast epithelial tissue and in tumorigenic tissue samples. Human breast tumor samples from patients undergoing surgery were obtained from Dr. Beth Schachter. The samples were categorized by hospital number.

Six different breast tissue samples (0.2 - 0.5 g of each) were homogenized in 10 ml of 0.25 M sucrose, 5 mM imidazole, pH 7, and 0.1% ethanol by means of 50-100 stokes of a homogenizer with a tight-fitting pestle. The homogenates were centrifuged for 30 min at 10,000 rpm in order to separate soluble from particulate components. The pellets were then resuspended in one tenth of the starting volume of buffered sucrose. Catalase and acyl-CoA oxidase were assayed as described [7,8], and protein concentrations were determined by the method of Bradford [9].

Results

The specific catalase activity measured using our standard assay was extremely low in both normal and tumor tissue. The levels for the tumor samples are shown in Table 1.

Table 1.

Sample	Catalase (units/mg protein)
89-48	0.0045
91-19	0.003
90-104	0.006
90-60	0.0065
91-43	0.006
91-63	0.002

These catalase levels are at the limits of the detection ability of the assay, thus we did not feel that this would be a reliable approach for comparing different tissue or cell samples. In addition we were unable to detect acyl-CoA oxidase activity in these samples using a highly sensitive assay [8]. For these reasons we decided to use a more sensitive approach in order to measure expression levels of genes encoding peroxisomal proteins in human samples (see below).

2) Quantitation of human peroxisomal enzyme gene expression using the polymerase chain reaction.

A polymerase chain reaction (PCR)-based method has previously been used to quantitate the expression levels of low abundance genes [10-12]. In this method RNA is first isolated from human samples or cultured cells and is subsequently converted to cDNA by reverse transcription using random hexamers. This DNA can then be amplified by PCR and the products generated can be quantitated by various means. While this method is extremely sensitive, one must be aware of the exponential nature of the polymerase chain reaction and the potential problems of using this method for quantitative purposes. Therefore we adopted the method of Horikoshi et al. in which the relative, rather than the absolute, levels of gene expression are determined by comparing the ratio of PCR product generated by amplification of the target DNA and an endogenous internal standard gene in separate reactions [12]. Samples of 1 μg of RNA from control cells were subjected to reverse transcription and the cDNA was then used in a polymerase chain reaction using primers designed to amplify regions of human ß-actin, catalase or acyl-CoA oxidase. The primers were end-labeled with γ ^{32}P-ATP using T4 kinase. Samples of the reaction mixtures were then electrophoresed through a 10% acrylamide gel. The gel was dried, exposed to a phosphorImager screen and analyzed using Imagquant software. Levels of catalase and acyl-CoA oxidase were normalized to levels of ß-actin for each sample.

Establishing linearity of the reaction

The increase in the amount of PCR product as a function of the starting amount of DNA is described by the equation:

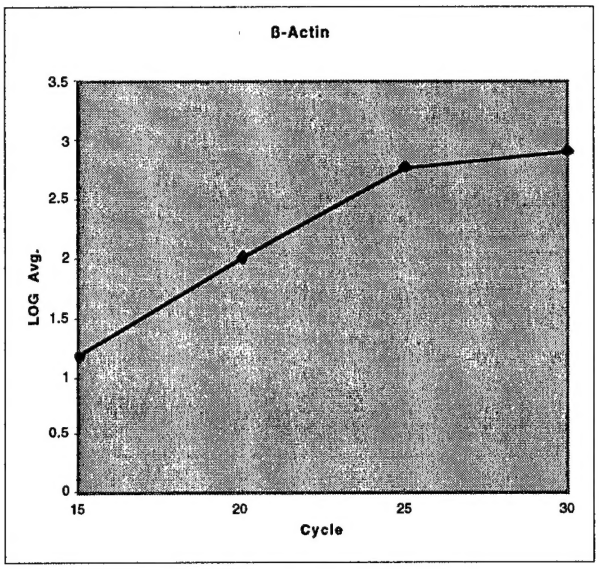
$$N = N_0 (1+eff)^n$$

where N_0 is the number of copies of the starting DNA
N is the amount of DNA after *n* cycles of amplification
eff is the efficiency of the amplification

[13].

According to this equation, even though the PCR reaction products increase exponentially, the amplified DNA at any value of *n* is linearly proportional to the amount of starting DNA. However, the efficiency of the PCR becomes lower with increasing extent of reaction due to consumption of primers and build-up of inhibitory phosphate levels. Therefore the linearity of the PCR product curves depend on the cycle number. In order to obtain an indication of the range of linear amplification for each gene of interest as a function of cycle number we performed a series of time course experiments. The resultant curves were linear in the middle region between 20 and 25 cycles in each case (Figure 1). All subsequent experiments were carried out using 25 cycles.

(a)



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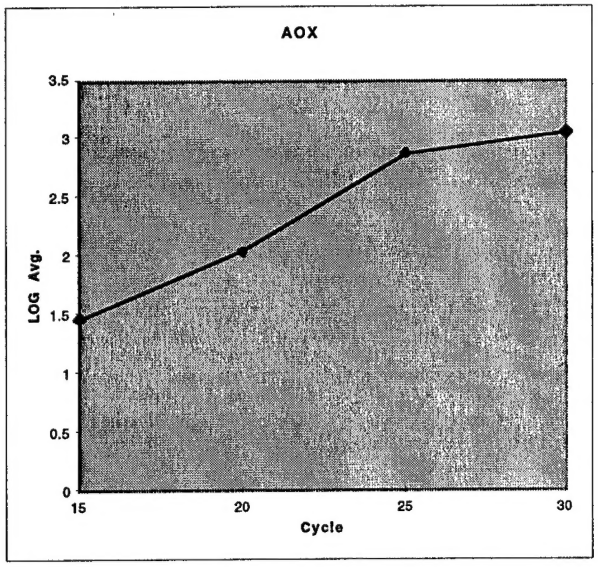

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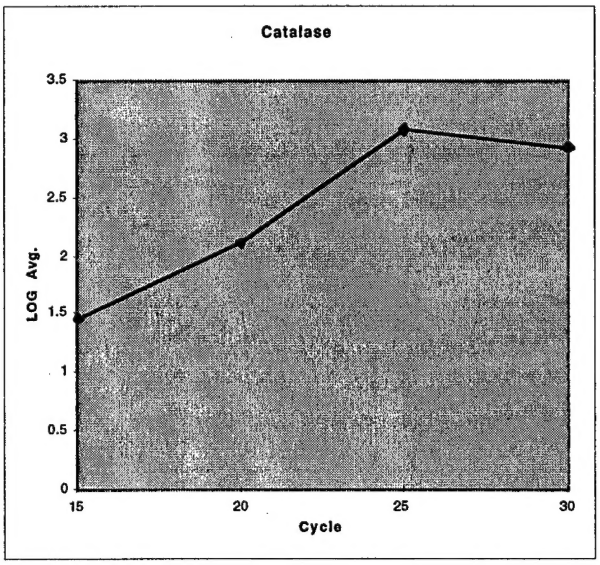

Figure 1. Optimization of the linearity curve for PCR of (a) actin, (b) acyl-CoA oxidase and (c) catalase cDNA. PCR products formed are plotted as a function of cycle number.

3) Use of reverse transcription PCR to measure the expression of genes encoding peroxisomal proteins in human breast tumor cell lines

The cell types shown in Table 2 were grown and harvested by Dr. Mira-y-Lopez who is collaborating with us on this project.

Table 2. Normal and carcinoma human breast cell lines used in this study.

Cells	Source
1) RM 12.5.94p2	Normal human breast cells derived from reduction mamoplasty
2) MT3V1-7	SV40 Immortalized normal luminal human breast epithelial cells
3) 184B5	Benzypyrene immortalized normal human breast epithelial cells
4) ZR75-1	Carcinoma cell line, estrogen receptor positive (ER+)
5) MDA-MB-468	Carcinoma cell line ER-
6) T47D	Carcinoma cell line ER+
7) BT20	Carcinoma cell line ER-
8) HS578T	Carcinoma cell line ER-
9) MDA-MB-231	Carcinoma cell line ER-

Approximately 5 X 10^6 cells were harvested by scraping and pelleting and were then snap-frozen at -80°C. Prior to use the cells were thawed and resuspended in 350 µl of lysis buffer. RNA was then extracted using a Qiagen RNeasy total RNA kit according to the protocol of the manufacturer. Approximately equal amounts of RNA were derived from each cell type (Figure 2).

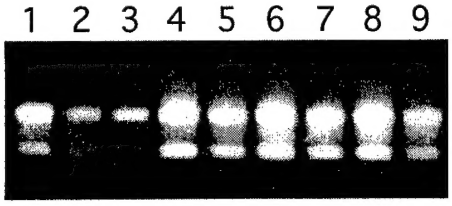

Figure 2. Ethidium bromide-stained gel of RNA isolated from cell samples 1 to 9 as shown in Table 2.

Samples of 1 µg of RNA were subjected to reverse transcription using the Gibco Superscript preamplification system. 10% of the reverse transcribed mixture was used as a template for each subsequent PCR reaction. The DNA primers for each reaction were designed to amplify fragments of ß-actin, hPPAR, catalase and acyl-CoA oxidase respectively and were labeled as described above. Initially we tested one control and one tumor cell line (numbers 1 and 7 from Table 2). PCR products of the expected size were visible on an ethidium bromide-stained gel for all fragments except hPPAR (Figure 3).

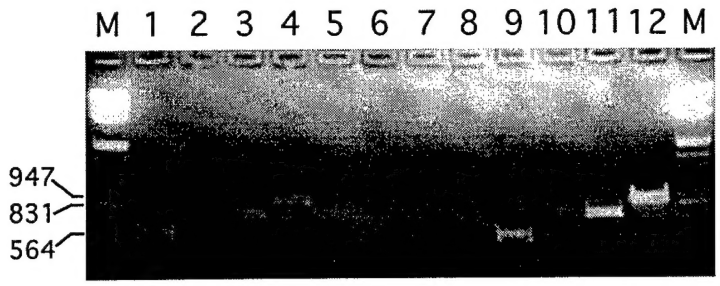

Figure 3. PCR products generated in the presence (lanes 1-4 and 9 to 12) and absence (lanes 5 to 8) of reverse transcriptase. PCR products were generated with primers to ß-actin (lanes 1, 5 and 9), hPPAR (lanes 2, 6 and 10), acyl-CoA oxidase (lanes 3, 7 and 11) and catalase (lanes 4, 8 and 12). The products were resolved in a 1% agarose gel and stained with ethidium bromide. M = molecular weight standards.

4) Comparison of acyl-CoA oxidase and catalase levels in human breast cell lines

The relative expression of acyl-CoA oxidase and catalase with respect to ß-actin was determined in three control samples (1 to 3 in Table 2) and two tumor cell lines (3 to 5 in Table 2) (Figure 4). The relative expression of acyl-CoA oxidase in control cells was fairly constant while the expression of catalase showed a three-fold variability (Table 3). The relative expression of both genes was higher in the tumor cell lines tested.

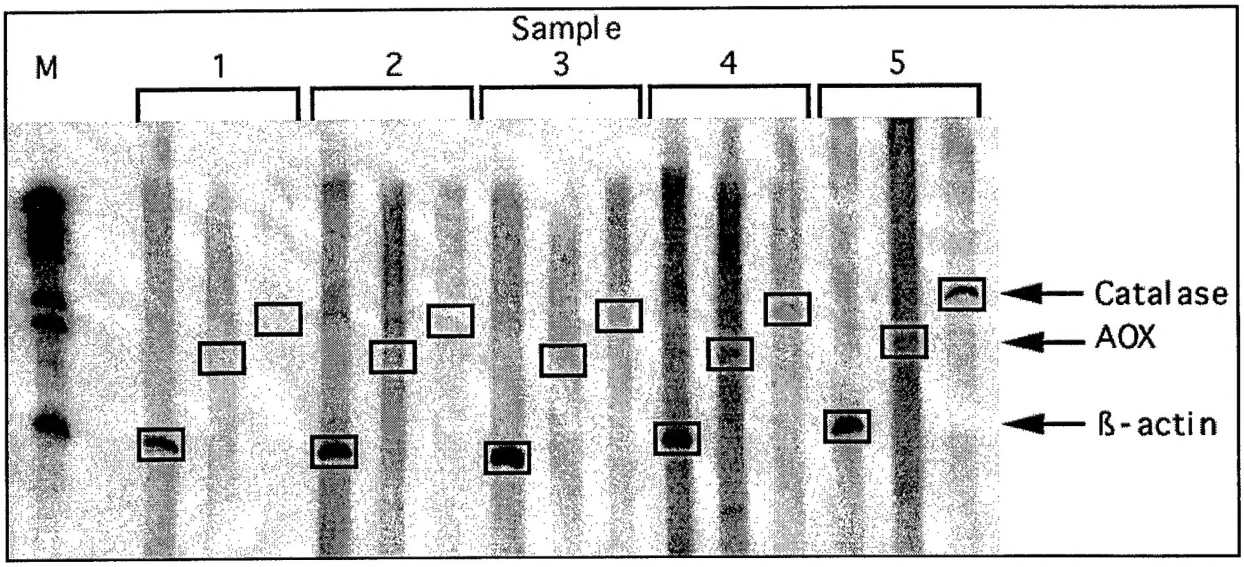

Figure 4. PCR products from amplification of ß-actin, acyl-CoA oxidase and catalase cDNA from samples 1 to 5 of Table 2. The PCR products were resolved in 10% polyacrylamide and the dried gel was exposed to a phosphorImager screen. M = radiolabeled DNA standards.

Table 3. Quantitation of PCR products shown in Figure 4.

Sample	Relative Aox expression	Relative catalase expression	Ratio Aox : catalase
1	17.4	5.8	3:1
2	23.6	8.46	3:1
3	16.25	18.05	1:1
4	45.2	21.15	2:1
5	45.3	52.7	1:1

We are currently examining the remaining cell lines we have in hand, and are gathering more samples in order to quantitate levels of catalase and acyl-CoA oxidase expression in the same manner.

Thus far we have been unable to detect hPPAR in these human samples. This is perhaps not surprising as this transcription factor is expressed at very low levels. For this reason we will adopt a more sensitive method, in which radiolabeled nucleotides are included in the PCR reaction mixture so that the radioactive signal is amplified [14]. We will also use this approach to amplify ß-actin so that the results obtained for hPPAR can be normalized in the same manner described above for catalase and acyl-CoA oxidase. By these means we should be able to compare the levels of this receptor in human normal and tumor cell lines and tissue samples.

Conclusions

Our studies to date have demonstrated that the levels of peroxisomal catalase and acyl-CoA oxidase in human breast tumor samples are very low, and outside the limit of quantitative analysis by standard assays. We have taken an alternative approach and have established conditions for accurate quantitation of gene expression using the polymerase chain reaction.

Our results with the small sample of cells that we have analyzed to date indicate that the ratio of acyl-CoA oxidase to catalase expression is similar in the normal cells to that found in the tumor cell lines. However, we cannot make any conclusions about these data until we have analyzed a larger number of cell lines. We will proceed to test the remaining cell lines that we have in hand, and will now also use this sensitive method to measure the expression of these genes in the human tumor samples that we have. In addition we will compare the levels of expression of hPPAR in each of these samples.

In summary we have made significant progress in establishing quantitative conditions to achieve Objectives 1 and 2 of our original statement of work. Within the next month we should be in a position to perform similar measurements on hPPAR expression (Objective 3).

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